CHAPTER 2: REVIEW OF LITERATURE

2.1. Introduction to Human papillomavirus and cervical cancer

Worldwide, cervical cancer is the fourth most frequent type of cancer in women, and causes an estimated 270,000 deaths every year.\textsuperscript{87} Whereas, especially in young women, it is one of the most common cause of death.\textsuperscript{16} As per population based large studies on genital Human papillomavirus (HPV) infection and cervical cancer, it has been indicated that, a variety of factors interplay in the attainment of this infection. However, it is shown that, the high risk HPV infection has an important role in progression of infection to cervical cancer, but may not be the only cause for this disease.\textsuperscript{13}

Cervical cancer depends on a variety of additional factors, that are associated with HPV infections.\textsuperscript{13} Persistent high risk HPV infections are associated with CIN,\textsuperscript{88} that are graded into CIN 1, CIN 2 and CIN 3, depending upon the severity of the lesion. However, other than persistent HPV infection, factors that increase the risk of HPV infection are: younger age, early sexual debut, multiple sexual partners, hormonal contraception, other sexually transmitted infections, multiparity, smoking, immunosuppression, and poor nutrition.\textsuperscript{89} Approximately, half of the HPV infections persists for a minimum duration of 6–12 months. The persistence of HPV infection could be defined as, the HPV positivity at two or more intervals of time, where, it is not required that the same HPV type be detected at consecutive visits (non-type-specific persistence), or the type specific persistence during the course of infection.\textsuperscript{90}

A systematic review, on the recurrence of new HPV infection even after completion of the treatment, reported that, the new HPV infections act as a potential risk factor for the development of precancer and cervical cancer.\textsuperscript{91} Thus, proving that, if a women once infected with the HPV and not screened properly at different intervals during her life time, is always more susceptible for getting new HPV infection, throughout her lifetime. However, noncompliance with cervical cancer screening, and diagnostic programs is one of the major hurdles faced by the clinicians. The noncompliance of subjects could be
because of the following reasons, especially with regard to transportation, child care, self-pay costs, education and health care knowledge, or employment constraints.\textsuperscript{92}

Along with the various risk factors, reproductive factors were also found to be associated significantly higher, with the incidence of HPV infection.\textsuperscript{93} Knowledge is still required to find the association between the two, so that, strong preventive methods can be developed, for the improvement of the strategies. However, a study on vertical transmission of HPV infection to the new born have reported that, 6 months post delivery, there was absence of persistent HPV infection. Which, suggested that, if the new born is delivered through an infected cervix, there is a probability of only temporary infection of HPV DNA.\textsuperscript{94}

Faridi \textit{et al.}, (2011), \textsuperscript{95} in their study have mentioned that, in the next few years, cervical cancer is going to be one of the most frequent causes of death, more so, in young females. According to WHO statistics, every year roughly 5,00,000 new cases, are being registered, out of which 2,50,000 are fatal. The most recent reports from USA showed that, women are more prone to this infection, than the men. The overall reported percentage of getting infection in women, irrespective of races, was as high as 17.9%, while the chances of men getting infection is as low as 8%.\textsuperscript{95} About 80\% of the women live in developing countries that do not have adequate cervical cancer screening, or treatment programs. Most developing countries have, insufficient infrastructure for cytology-based screening, and lack well-trained cytopathologists. Thus, only a minority of women receive effective screening, and treatment for cervical cancer.

The hybrid capture 2 (HC2) assay, can detect 13 types of high-risk HPVs, associated with cervical cancer: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. However, this assay is costly, and needs sophisticated instruments, which are not easily available in poor resource countries.\textsuperscript{96} Using hybrid capture chemiluminescence technology, Qiagen has developed a kit for the detection of high risk HPV DNA, known as careHPV test, which has many advantages like: it is a powerful, rapid, and precise in HPV DNA detection. In which, antibodies bind to magnetic beads, rapidly capturing specific target HPV nucleic acid sequences, which are then detected using a chemiluminescence signal.\textsuperscript{96} This method
can detect 14 types of high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and more than 80 specimens can be processed in 2 hours. This allows screening, and follow-ups to be completed in one day.96,97

2.2.1. History of Human papillomavirus infection

While reviewing the exfoliative cytology of the viral wart, condyloma acuminatum, Meisels and Fortin, (1976), wrote, "no clear cytological pattern has yet been identified which would permit the unequivocal identification of condyloma acuminatum, from the cellular sample”. The cytological description includes dyskeratotic cells, bi or multinucleate cells, and balloon cells (which had a characteristic prominent perinuclear clearing or "halo", which failed to stain with eosin or periodic acid schiff).98

Human papillomavirus (HPV) infection is diagnosed by detection of viral DNA, but not by isolation of virus. Among various types of infections caused by HPV, the genital infection is predominantly, but not exclusively, a sexually transmitted infection. Penetrative vaginal or anal intercourse is not a necessary prerequisite for acquiring the infection by this virus, because it can be transmitted by direct contact with skin or mucosa, during intimate contacts of the genitalia or other mucosal surfaces infected.99 HPV being highly infectious has an incubation period ranging from 3–4 weeks to months or years. However, the duration of this latency probably relates to the viral load.100

Eventually, for reasons, not yet very well understood, the cell becomes permissive and viral growth commences, later, infectious virus is produced and released. This phase of active replication also remains for a variable length of time, but eventually, majority of infected individuals, develop an effective immune response, and become viral DNA negative.101 Consistent immunity depends on the cell-mediated effective response, to the early proteins, mainly E2 and E6. This kind of immune response promotes the regression of the lesion, accompanied, or followed by seroconversion with neutralizing antibody production for the major capsid protein L1.102
2.2.2. History of cervical cancer

In the year, 1842, Domenico Rigoni Stern, a physician from Italy, published a series of mortality statistics of women, dying of cancer in the city of Verona. In his demographic analysis, he reported that, uterine cancer was much more common in married women, and widows than, in unmarried women (for e.g., nuns and virgins). This was the first report that suggested uterine cancer was somehow related to sexual activity.

Later, in the year 1908, Walther Schauenstein, an Austrian gynecologist, proposed the concept that cancer of uterine cervix was preceded by precancerous states, limited to the epithelium. This concept was essential for the cervical cancer prevention programmes, which were based on recognition and elimination of precancerous states, thus, preventing invasive cancer. Screening by cytology, was recognized by George Papanicolaou, in 1928, and the technique became the method for the identification of precancerous states. However, in the same year, Herbert Traut, provided papanicolaou with vaginal smears, which was later published in 1941. This was the beginning of the ‘Pap smear’.

Direct sampling of the uterine cervix was rediscovered in 1949, by J Ernest Ayre (Canadian gynecologist/cytologist). Among the many varieties of cells observed in the cervical smears, there were some that were characterized by large nuclei, and with large clear perinuclear spaces. These cells were thoroughly studied by Ayre, who called them ‘halo cells’ and proposed that they are precursors of cervical cancer. In 1960, he proposed that, the ‘halo cells’ may correspond to a viral infection in precancerous states.

The term, koilocytes, was derived from the term ‘koilocytotic atypia’, proposed by Koss and Durfee in 1956. The histologic equivalent of koilocytes was named ‘warty atypia’, because of its resemblance to warts or condylomas. Meisels and Fortin from Canada (1976), proposed that the koilocytes may be a link to condylomas, hence, indicating their viral origin of cervical cancer. The first documentation that the nuclei of koilocytes contained viral particles, was presented by Laverty et al., (1978), from Australia.
2.3. Morphology and molecular structure of Human papillomavirus

Morphology

Human papillomaviruses (HPV) are small, non-enveloped, icosahedral viruses and measures about 52–55 nm in diameter (Fig. 2.1). The viral particles consist of a double-stranded DNA molecule, which is made up of about 8,000 base-pairs (bp). The virus is enveloped in a protein capsid composed of 72 pentameric capsomers, which contains two structural proteins - late L1 (55 kDa in size; 80% of total viral protein) and L2 (70 kDa). The production of virus-like particles (VLPs) is possible by the expression of L1 alone, or in combination with L2. The intact virion has a density of 1.34 g/mL in cesium chloride and a sedimentation coefficient (S20, W) of 300.  

Fig. 2.1 Structure of Human papillomavirus
(This picture is adopted from: http://soundprint.org/getImage/ID/162/HPV.jpg)
Molecular Structure of virus

A closed, double-stranded 8-kbp DNA molecule, that comprises the HPV genome has early and late genes, clustered in separate regions as shown in Fig. 2.2. Early genes code for proteins, involved in viral DNA replication, transcription control, and cellular transformation. The major viral capsid protein (L1), and a minor capsid protein (L2) are encoded by the late genes. In between these two regions, there is upstream regulatory region (URR), also known as the long control region. This non-coding region contains promoters and elements involved in DNA replication and transcription. Each HPV type not only has the promoter for E6, common to all HPVs, but also one or more specific promoters in the URR. \(^{114,115}\)

Fig. 2.2 Linear schematic diagram of papillomavirus genome

It is a closed circle of double-stranded DNA but is linearized for convenience of alignment. Boxes show protein coding regions. Regulatory region regulates viral gene expression and viral DNA replication. Differences exist in protein function among different viral genotypes. In general, E6, E7, and E5 are transforming genes, E1 and E2 coordinate viral genome replication and expression, and L1 and L2 are structural proteins, forming viral capsids. E4 may actually be a late protein involved in virus release from the cell’s keratin framework. This figure is adopted from Tyring et al., (2000). \(^{114}\)
2.3.1. Life cycle Human papillomavirus

After getting entry through microwounds of the epithelium, the HPV life cycle begins with the infection of stem cells, in the basal layer of the epithelium, the only tissue in which they replicate. The virus infects the epithelial tissues through micro-abrasions, or other epithelial trauma, that exposes the basement membrane. For initiation of transcription, the infectious process takes about 12–24 hours. It is believed that, the neutralizing antibodies play a major role, while the virions still reside on the basement membrane and in cell surfaces.

Once inside the cells, to maintain a low number of copies of the genome, HPV requires the expression of E1 and E2 genes. These proteins bind to the viral origin of replication, and recruit cellular DNA polymerases and other proteins necessary for DNA replication. In the suprabasal layer, the expression of genes E1, E2, E5, E6, and E7 contributes to the maintenance of the viral genome, and induces cell proliferation, by increasing the number of HPV infected cells in the epithelium, resulting in a higher number of cells, that will eventually produce infectious virions. Occurring in the more differentiated cells of the same layer of the epithelium, is the activation of the differentiation dependent promotor, and maintenance of the gene expression of E1, E2, E6, and E7.

In addition, there is activation of the expression of the E4 gene, whose product will induce amplification of the viral genome replication, which greatly increases the number of virus copies per cell, while, simultaneously, there is expression of genes L1 and L2. In the granular layer, the products of late genes, the major and minor proteins of the viral capsid, L1 and L2, respectively, gather to assemble the viral capsid and formation of new virions, which reach the cornified layer of the epithelium, and are released.
2.3.2. Immune response to the host

Serological assays have the potential of being more accurate, sensitive, and relevant for the detection of disease progression, compared to direct HPV DNA detection in gynecologic smears. Around 80% of women get infected at some stage of their life, as high-risk HPV infection is common. But as a result of this infection cervical cancer arises very rarely. As a result of cell-mediated immune response, most infections are self cleared, although, HPV 16 and 18 persist longer than other high-risk types. Regression of anogenital warts is accompanied by, a CD4+ T cell-dominated Th1 response, which is also seen in animal models of papillomavirus disease. Failure to develop an effective cell-mediated immune response, results in persistent high-risk HPV infection, which in turn, increases the probability of progression towards invasive carcinoma.

Competent cell-mediated surveillance is required for the control of HPV infection, as immunosuppression or immunocompromising diseases increase the incidence of genital
HPV infections, and HPV associated diseases. Mononuclear-cell inflammatory infiltrates are the major morphological feature in regressing skin warts.\textsuperscript{120}

2.3.3. Human papillomavirus types

De Villiers \textit{et al.}, (2004), described the taxonomy of Human papillomaviruses (HPV), as HPV 1 to HPV 96. Since, HPV 46, 55, 64 and 79 did not meet the criteria, they were omitted, and their numbers left vacant.\textsuperscript{128} There are, Five Alpha PVs: type 97, 102, 106, 114, and 117; 14 Beta PVs: type 98, 99, 100, 104, 105, 107, 110, 111, 113, 115, 118, 120, 122, 124; and nine Gamma PVs: type 101, 103, 108, 109, 112, 116, 119, 121, 123. Among the nine gamma PVs, HPV types 101, 103, and 108, differ from all other HPV types, in that, they lack an E6 ORF.\textsuperscript{129, 130} In spite of this distinction, these three types are included in the genus Gamma PV, based on the current rules of sequence similarities in the L1 ORF and the resulting topology of the phylogenetic tree.\textsuperscript{8}

The anogenital tract is mainly affected by the subtypes: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66 and 69.\textsuperscript{7} Further, these specific subtypes are classified as, high-risk and low-risk types on the basis of their oncogenic potential. These subtypes hold a remarkable ability, to cause neoplastic changes and have an important role in development of a variety of malignancies.\textsuperscript{8} The HPV type 6 and 11 are the low risk genital types, while, on the other hand HPV 16 and 18 fall under the high risk genital types.\textsuperscript{9}

2.3.4. Early and late proteins in Human papillomavirus infection

Early genes

The early genes include E1, E2, E4, E5, E6, and E7. The E1 and E2 genes code for important regulatory proteins of HPV. The function of these two proteins is critical for DNA replication, and permissive infection. The host cells provide DNA polymerase, and associated DNA-replicating proteins. The E2 protein represses transcription by binding to E2 binding sites in the upstream regulatory region (URR).\textsuperscript{131} The protein coded by E1, facilitates the binding of E2 protein, in the promoter region. The E6 and E7 proteins are important in host DNA synthesis.
The E2 protein is a phosphoprotein, with three functional domains. The N-terminal domain contains approximately 220 amino acids, and acts as a transactivator. The second domain, the C-terminal, contains about 90 amino acids and, in its dimeric form, can bind to DNA. The third domain is the hinge region between the other two domains.\textsuperscript{114}

**Late genes**

The two late genes, L1 and L2, encode the major and minor capsid proteins, respectively; these are the structural proteins of the virion.\textsuperscript{132} E4 is probably a late gene, because it is expressed late in the cycle of virus replication. In addition, E4 may be involved in disrupting the structure of the host cell cytoskeleton, thus, facilitating the assembly and maturation of the virus.\textsuperscript{133} The HPVs are classified by nucleic acid homology, either by hybridization, or by direct sequence comparison. To be considered a new type, a HPV must exhibit less than 90\% homology to known types, in the L1 and E6 genes, as well as in the upstream regulatory region (URR). If homology falls in the range of 90-98\%, the virus would be considered a subtype. If the homology is >98\%, the virus would be considered a variant.\textsuperscript{114}

**E6/E7 proteins in cervical cancer**

Among eight genes encoded by HPV, two early genes, i.e., E6 and E7, play a major role in the development of malignancies.\textsuperscript{134} E6 binds to P53, and interferes with its translocation, and thereby inhibits the ability of P53 to activate or repress target genes. Whereas, E7 binds to hypophosphorylated Rb, and thereby induces cells to enter into premature S-phase by disrupting Rb-E2F complexes.\textsuperscript{14} In humans, the oncogenes, E6 and E7, are invariably expressed in uterine cancers, and their continued expression is a prerequisite for maintenance of the cancerous state.\textsuperscript{135} Table 2.1. shows the functions of all early and late proteins of HPV.
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>DNA-binding functions and a binding site in the origin of replication localized in the long control region, a prerequisite for viral DNA replication</td>
</tr>
<tr>
<td>E2</td>
<td>Controls viral transcription, DNA replication, and segregation of viral genomes</td>
</tr>
<tr>
<td>E4</td>
<td>Favors and supports the HPV genome amplification, regulating the expression of late genes, controlling the virus maturation, and facilitates the release of virions</td>
</tr>
<tr>
<td>E5</td>
<td>Enhances the transforming activity of E6 and E7; promotes fusion between cells; contributes to immune response evasion</td>
</tr>
<tr>
<td>E6</td>
<td>Binds and degrades the tumor-suppressor protein p53, inhibits apoptosis; interacts with proteins of the innate immune response</td>
</tr>
<tr>
<td>E7</td>
<td>Binds and degrades the tumor-suppressor protein pRB; affects the expression of S-phase genes by directly interacting with E2F factors; induces a peripheral tolerance in cytotoxic T lymphocytes; down regulates the expression of TLR9, contributing to immune response evasion</td>
</tr>
<tr>
<td>L1</td>
<td>Major capsid protein; contains the major determinant required for attachment to cell-surface receptors; highly immunogenic and has conformational epitopes that induce the production of neutralizing type-specific antibodies against the virus</td>
</tr>
<tr>
<td>L2</td>
<td>Minor capsid protein; contributes to the binding of virion in the cell receptor, favoring its uptake, transport to the nucleus, and delivery of viral DNA to replication centers</td>
</tr>
</tbody>
</table>

*E1 to 7 and L1 and L2: Early and late proteins in HPV infection*
2.3.5. Mucosal and cutaneous Human papillomavirus infections

A. Mucosal HPV infections:

*Condyloma acuminatum* is one of the commonest manifestations of Human papillomavirus (HPV) in the genital area. The presentations of the disease can be as papules, nodules or soft, filiform, sessile growths. The disease is usually sexually transmitted, and is most frequently caused by low-risk HPVs, such as HPV 6 and 11, although many other genotypes can also be found.\(^\text{136-138}\)

*Focal epithelial hyperplasia* is a rare manifestation of HPV related disease, of the oral mucosa which, commonly affects, children and women. Lesions are mainly located in the lower lip, but less frequently may affect the upper lip, tongue, oral mucosa, oropharynx, palate, and floor of mouth. HPV 13 and 32 are mainly responsible for this disease.\(^\text{139}\)

*Cervical neoplasia and cervical cancer:* Precancerous cervical lesions are classified as, cervical intraepithelial neoplasia (CIN) of different grades (1, 2, or 3). Productive viral infection is evident by the low-grade lesions, with the presence of koilocytes in the suprabasal cell layers. HPV is detectable in 90–100% of cervical abnormalities, ranging from early cytological abnormalities and dysplasia, to frank cervical cancer.\(^\text{140-142}\)

*Other anogenital cancers:* including those of the vulva, vagina, penis, and anus. HPV prevalence is 90% in vulvar intraepithelial neoplasia, vaginal or warty cancers, but is rare in keratinizing squamous cell carcinomas.\(^\text{143, 144}\) Most prominent type in vulvar cancer is HPV 16. Anal cancer is more common in homosexual men and in immunosuppressed populations.\(^\text{115}\)

*Head and neck cancer:* A recent meta-analysis showed that, HPV prevalence in head and neck squamous cell carcinoma (HNSCC) increased significantly from 41% in 2000 to 72% in 2004.\(^\text{145}\) Association of HPV is now a part of routine diagnostic procedure, while assessing the prognosis of HNSCC. HPV 16 is the most common type found in HNSCC, but other HPV types such as 18, 31, 33, and 35 can also be detected.\(^\text{145}\)
B. Cutaneous HPV infections

Common warts can be single or multiple with varying sizes. Most often, they appear on the back of hands, however, knee being a common site of infection in children.\textsuperscript{146, 147} HPV 1, 2, 4, 27, and 57, are the most prevalent types,\textsuperscript{148-150} whereas, HPV 7 is commonly associated in common warts of individuals whose hands are constantly exposed to moisture and cold.\textsuperscript{151}

Plantar warts occur on the soles of the feet, particularly among children. Common HPV types which are responsible for these warts are: type 1 and 4. Although, HPV 57, 60, 63, 65 and 66, can also be a cause of infection.\textsuperscript{152} HPV 1 commonly induces lesions, that manifest as a keratotic plug surrounded by a hyperkeratotic rim, that are often painful. HPV 4 can be the cause of mosaic warts, which are more superficial lesions, which occur in a confluent, cobblestone pattern, and are usually painless.\textsuperscript{153}

Flat warts are slightly raised lesions over the skin. These warts are pigmented, flat, and smooth or with slightly rough surface. Commonly affected sites are face and back of hands. HPV 3 and 10 are the most common types responsible for this wart.\textsuperscript{146, 154}

Pigmented warts range from gray to blackish brown in color. Mostly, they are located on the palmoplantar or lateral surfaces of the hands, feet, fingers, and toes. HPV types 4, 60, and 65 are the frequent types associated in such lesions.\textsuperscript{155}

Skin cancer is a squamous cell carcinoma (SCC) \textit{in situ} of the skin. The link between HPV and non-melanoma skin cancer is not clear. Mucosal HPV types, especially HPV 16 can sometimes be detected in skin cancer, but also very rarely HPV 2, 31, 34, 35, 58, 61, and 73.\textsuperscript{156, 157}

C. Cervical cancers

Cervical cancer accounts for 10\% of all female cancers, making it the one of the leading cause of cancer death in women.\textsuperscript{158} Important etiological factors in the development of this cancer are, HPV types - 16, 18, 31, 33, 35, etc.\textsuperscript{158, 159} Cervical cancer can be suspected on cytological examination of exfoliated cervical cells, as it is characterized by
a well defined, pre-malignant phase, which can be confirmed on histological examination of cervical material. These pre-malignant changes ranging from CIN 1 (mild dysplasia) to CIN 2 (moderate dysplasia) to CIN 3 (severe dysplasia / carcinoma in situ).\(^1\)

### 2.3.6. Classification of cervical cancer

The Papanicolaou classification consisted of five classes, i.e., I to V. The Reagen system, later adopted by the WHO, which divided abnormalities into mild, moderate and severe dysplasia, and carcinoma-in-situ (CIS).\(^{160}\) Later, in a classification system by Richart, (1980), same terminology is used, as the histological changes with different grades of cervical intraepithelial neoplasia grading from CIN 1 to 3.\(^{161}\) More recently, according to the Bethesda system, (2001), lesions were classified as low or high grade squamous intraepithelial lesions (LGSIL or HGSIL).\(^{162}\) However, this classification also includes, one group of lesions, characterized as “atypical squamous cells of undetermined significance” (ASCUS). Table 2.2. shows the different classification systems for cervical cytology.

**Table 2.2 Systems for classifying cervical cytology**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Classification</th>
<th>Severity of the cervical pathology based on cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bethesda</td>
<td>Normal Infection Reactive Repair ASCUS Squamous Intraepithelial Lesions (SIL) Invasive carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low grade (LSIL) High Grade (HSIL)</td>
</tr>
<tr>
<td>2.</td>
<td>Richart</td>
<td>Normal Condyloma Cervical intraepithelial Neoplasia (CIN)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIN 1 CIN 2 CIN 3</td>
</tr>
<tr>
<td>3.</td>
<td>Reagen</td>
<td>Negative Atypia Mild Dysplasia Moderate Dysplasia Severe Dysplasia Carcinoma in Situ (CIS)</td>
</tr>
<tr>
<td>4.</td>
<td>Papanicolaou</td>
<td>I II III IV V</td>
</tr>
</tbody>
</table>

*Abbreviations used: ASCUS: Atypical squamous cell of undetermined significance, LSIL: Low grade squamous intraepithelial lesion, HSIL: High grade squamous intraepithelial lesion.*
2.3.7. Presentation, clinical features and staging of cervical cancers

The precancerous lesions, i.e., CIN are asymptomatic and usually detected through screenings, or in a pelvic examination. Invasive disease can also be asymptomatic, but one of the most common symptom is abnormal vaginal or post-coital bleeding, as well as, increased vaginal discharge.\textsuperscript{163} Symptoms of more advanced stages of the disease include, pelvic pain (resulting from tumor extending into the pelvic wall), haematuria (resulting from pressure on the bladder), or constipation (from the pressure on the rectum).

The most common diagnostic test for cervical cancer is cervical cytology, which is used as a screening test. If the cytology result is abnormal, or if there are other indications, then other more invasive investigations, such as colposcopy, endocervical curettage, and directed biopsy should be carried out to confirm the diagnosis.\textsuperscript{164}

Cervical cancer staging

Cervical cancer is a clinically staged disease. The FIGO staging system\textsuperscript{165} is the current standard, and is applicable to all histologic types of cervical cancer. Table 2.3 shows the current FIGO staging system of cancer.

\textit{Table 2.3 FIGO staging of cancer of cervix uteri} \textsuperscript{165}

<table>
<thead>
<tr>
<th>Stage</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Carcinoma strictly confined to the cervix; extension to the uterine corpus should be disregarded.</td>
</tr>
<tr>
<td>IA</td>
<td>Invasive cancer identified only microscopically. Invasion is limited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm</td>
</tr>
<tr>
<td>IA1</td>
<td>Measured invasion of the stroma no greater than 3 mm in depth and no wider than 7 mm diameter.</td>
</tr>
<tr>
<td>IA2</td>
<td>Measured invasion of stroma greater than 3 mm but no greater than 5 mm in depth and no wider than 7 mm in diameter.</td>
</tr>
<tr>
<td>Stage</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
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</tr>
<tr>
<td>IB</td>
<td>Clinical lesions confined to the cervix or preclinical lesions greater than Stage IA. All gross lesions even with superficial invasion are Stage IB cancers.</td>
</tr>
<tr>
<td>IB1</td>
<td>Clinical lesions no greater than 4 cm in size.</td>
</tr>
<tr>
<td>IB2</td>
<td>Clinical lesions greater than 4 cm in size.</td>
</tr>
<tr>
<td>Stage II</td>
<td>Carcinoma that extends beyond the cervix, but does not extend into the pelvic wall. The carcinoma involves the vagina, but not as far as the lower third.</td>
</tr>
<tr>
<td>IIA</td>
<td>No obvious parametrial involvement. Involvement of up to the upper two-thirds of the vagina.</td>
</tr>
<tr>
<td>IIA1</td>
<td>Clinically visible lesions &lt;4.0 cm in greatest dimension.</td>
</tr>
<tr>
<td>IIA2</td>
<td>Clinically visible lesions &gt;4.0 cm in greatest dimension.</td>
</tr>
<tr>
<td>IIB</td>
<td>Obvious parametrial involvement, but not into the pelvic sidewall.</td>
</tr>
<tr>
<td>Stage III</td>
<td>Carcinoma that has extended into the pelvic sidewall and/or involves the lower third of the vagina. Cass hydronephrosis or a non-functioning kidney.</td>
</tr>
<tr>
<td>IIIA</td>
<td>No extension into the pelvic sidewall but involvement of the lower third of the vagina.</td>
</tr>
<tr>
<td>IIIB</td>
<td>Extension into the pelvic sidewall or hydronephrosis or non-functioning kidney.</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Carcinoma that has extended beyond the true pelvis or has clinically involved the mucosa of the bladder and/or rectum.</td>
</tr>
<tr>
<td>IVA</td>
<td>Spread of the tumor into adjacent pelvic organs.</td>
</tr>
<tr>
<td>IVB</td>
<td>Spread to distant organs.</td>
</tr>
</tbody>
</table>
2.3.8. Pathology of cervical cancer

There are two main types of cervical cancer: squamous cell carcinoma (SCC), and adenocarcinoma (ACC). SCC is the most common type of cancer, and accounting for about three quarters of all cases. It develops from the transformation zone, which is located at the junction between the squamous and columnar cells of the cervix (squamocolumnar junction), which migrates from the exocervix to the distal endocervical canal, with advancing age. The second type of cervical cancer is ACC, which develops from the mucus producing cells of the endocervix, accounts for approximately 18% of cervical carcinomas. The other cervical carcinomas are adenosquamous (4%) and other carcinomas (5%) or malignancies (1.5%).

Common pathological types

- **Squamous cell carcinoma**

  Invasive squamous cell carcinoma (SCC) is the most common variety of invasive cancer in the cervix. Histologically, variants of SCC include, large cell keratinizing, large cell non-keratinizing, and small cell types. Large cell keratinizing tumors consist of tumor cells forming irregular infiltrative nests, with laminated keratin pearls in the center.

- **Adenocarcinoma**

  In recent years, there is an increasing number of cervical adenocarcinomas reported in women in their 20s and 30s. Although the total number of cases of adenocarcinoma has been relatively stable. This cancer is more common in young women, especially as the number of cases of invasive SCC decreases.

- **Adenosquamous carcinoma**

  Adenosquamous carcinomas are mixture of malignant glandular and squamous components. Patients with adenosquamous carcinoma of the cervix are reported to have a poorer prognosis, than those with pure adenocarcinoma or squamous carcinoma.
- **Sarcoma**

The most important sarcoma of the cervix is embryonal rhabdomyosarcoma, which occurs in children and in young adults.\textsuperscript{167}

- **Malignant melanoma**

On rare occasions, melanosis is seen in the cervix. Thus, malignant melanoma may arise in this area. Histopathologically, it simulates melanoma elsewhere, and the prognosis depends on the depth of invasion into the cervical stroma.\textsuperscript{167}

### 2.4. Associated Human papillomavirus cancers

Although cervical cancer is the most common type of cancer associated with HPV, there is increasing evidence that, HPV is an important etiologic agent in several less prevalent, but equally important types of cancer. Specifically, studies suggest that, 90\% of anal, 65\% of vaginal, 50\% of vulvar, and 35\% of penile cancer cases are associated with oncogenic type HPV infections.\textsuperscript{168,169} Additionally, data suggest that, approximately 60\% of the people who develop oropharyngeal cancer are infected with at least one oncogenic subtype of HPV. Interestingly, a geographical correlation between incidence of anal, vulvar, vaginal, penile, and head and neck cancers has been observed.\textsuperscript{170,171}

### 2.5. Respiratory papillomatosis

Recurrent respiratory papillomatosis is a relatively rare disease, mainly caused by low-risk HPV types, primarily by types 6 or 11. Although, this disease has a worldwide distribution, it is more prevalent in some countries, and areas than in others.\textsuperscript{68} Juvenile and adult forms have been described, and both boys and girls appear to be nearly equally affected by juvenile onset recurrent respiratory papillomatosis. Juvenile onset recurrent respiratory papillomatosis is believed to result from HPV transmitted from mother to infant during delivery. In contrast, with adult-onset recurrent respiratory papillomatosis, this preferentially affects men more than women, at a ratio of approximately 3:2.\textsuperscript{112}
2.6. **Human papillomavirus infection in immunocompromised patients**

Studies show that, the chance of Human papillomavirus (HPV) infection, and HPV associated disease are high in individuals with immunosuppression, and or are HIV positive. The incidence and persistence of HPV infection, both are associated with the high HIV RNA levels, and CD4 count \(<200/mm^3\).\(^{172}\) It is found that, the chance of development of squamous intraepithelial lesion is more likely among women, with oncogenic HPV, as well as HIV positive with low CD4 cell count, as compared the women who are either HIV negative or with high CD4 cell count.\(^{173}\) There is very limited data available on the effect of highly active anti-retroviral therapy on HPV infection and its associated diseases. Therefore, further investigations are required in this area in future.\(^{174}\)

2.7. **Oral infection**

Benign lesions of oral cavity are associated with numerous HPV types, including subtypes 1, 2, 4, 6, 7, 11 and 13. Oral HPV related benign verrucal-papillary lesions are clinically subdivided into, verruca vulgaris, condyloma acuminatum, multiple and single papillomas, and focal epithelial hyperplasia.\(^{112}\) HPV 2 and 4 are responsible for verruca vulgaris, and HPV 6 and 11 associated with condyloma acuminatum and oral squamous papillomas. Studies have detected HPV capsid in 10% of oral condylomatous and 22% of hyperkeratotic papillomas.\(^{112}\) Patients with genital condyloma has a high incidence of HPV induced oral lesions. Up to 50% of individuals with widespread genital condyloma have oral condyloma acuminatum.\(^{112}\)

2.8. **Risk factors associated with the Human papillomavirus infection**

A number of risk factors are associated with the development of cervical cancer. Only a few of these factors have been examined in connection, with genital HPV infection so far. Majority of the studies are unable to clearly pinpoint the scope of action of the risk factor examined in relation to HPV infection.\(^{120}\)
Sexual behavior

The number of sexual partners is recognized, as an independent risk factor for the acquisition of genital HPV infection, and is probably not associated with events which transform HPV infection to HPV associated neoplasia. More than 40% young women are infected with HPV, during the first 2 years after initiation of sexual activity. Adolescents, and, sexually active women<25 years of age have a high risk of infection. The main reason why young women are more vulnerable to HPV infection, is biological. The cervical cells are not only more susceptible to HPV infection, but are also more prone to persistent HPV infection during ectopy. Ribeiro et al., (2015), and Baudu et al., (2014), found a significant association between, age at first sexual intercourse and HPV infection. More number of sexual partners also increases chances of coming into contact with a person who is carrying HPV. Some studies show that, women who had, two or more partners during their life, have twice higher prevalence of HPV than, those who had only one partner.

Circumcision

Many studies are done to identify the effect of circumcision, and it is found that circumcised men are less likely to get penile and prostate cancer. According to the CDC (Centers for Disease Control and Prevention), the foreskin is simply more sensitive to HPV infection than, the skin over the shaft. Another possible explanation could be that, the foreskin being susceptible to tearing during the intercourse, which might give viruses an easy entry inside the body. A study on HPV infection in men shows that, duration for clearance of any HPV infection was significantly longer, among circumcised men than, uncircumcised men. However, some research state that, male circumcision was not associated with an overall reduction in the incidence of genital HPV detection in men.

Immunosuppression

Human immunodeficiency virus (HIV) damages the immune system, and individuals infected with this virus, are at a higher risk for HPV infections as well. HPV is believed to be more dangerous among HIV positive individuals, due to the impact of HIV on cell-mediated immunity; a critical component required for clearance of HPV infection. The immune system plays a significant role in killing cancer cells, and thus, slowing their growth and spread. Women with
HIV or who are on immunosuppressive treatments (chemotherapy, monoclonal/polyclonal antibodies, glucocorticoids, etc.) might develop a cervical precancer into an invasive cancer faster than it normally would. Hence, because of lowered blood cells, especially white cells, women can be placed at a greater risk of infection.\textsuperscript{180}

**HIV and other infectious agents**

Individuals positive with HIV, have a high chance of HPV infections and HPV associated neoplasia.\textsuperscript{120} Cytological signs of HPV infection were found in women positive for HIV at a significantly higher rate, compared with women at risk for HIV infection or with women negative for HIV.\textsuperscript{120}

Based on literature available, \textit{C. trachomatis} infection for HPV positive women is associated with high grade CIN or cancer.\textsuperscript{182} Although, HPV infection of the cervix is not believed to be inflammatory, during \textit{C. trachomatis} infection, higher amounts of cytokines are secreted, which ultimately results in a severe inflammatory state. The inflammation effect of the \textit{C. trachomatis} infection, may lead to chronic cervical tissue damage, indirectly resulting from the production of reactive oxygen species, triggering an inflammatory cascade, decreasing cellular immunity, and promoting angiogenesis.\textsuperscript{182} Risk of cervical cancer is also increased by Herpes simplex virus-2 infection. This virus is among the several factors that, work in concurrence with HPV in boosting risk for cervical cancer.\textsuperscript{180}

**Age**

The highest rate of prevalence of HPV in gynecologic smears is in the age group of 20-24 years, with a decline as the age progresses.\textsuperscript{120} Since, the various HPV types have a similar pattern of distribution in all age groups, a cohort effect cannot explain this phenomenon.\textsuperscript{120}

**Oral contraceptives, hormones**

The oral contraceptives (OCs) used for a long time, are associated with cervical cancer diagnosis among HPV positive women. It was speculated that, use of contraceptives would possibly have an effect on clearance or persistence of HPV infection, progression or regression of pre-neoplastic and neoplastic lesions. According to literature, if a woman’s intake of oral contraceptives is high, then she is at a higher risk of developing cervical cancer and vice versa. However, a significant association of cervical cancer has been found with consumption of OCs over 5 years.\textsuperscript{180}

**Smoking**
The association of cigarette smoking and HPV prevalence, incidence, and persistence, is reported in many studies. In comparison to women who smoke vs non-smokers, women who smoke, are twice as likely as to get cervical cancer. However, in the recent past, smoking was identified as a risk factor for HPV detection in men, and it is reported to be associated not only with virus persistence, but also with anal and penile cancer. Smoking acts immunosuppressively on the cervix, by decreasing the population of Langerhans cell, and may alternatively or synergistically, promotes the acquisition of HPV infection. According to researchers, the substances and metabolites of smoke damages the DNA of cervical cells, which leads to an increase in cell proliferation, and may contribute to the development of cervical cancer. It is known that smoking also makes the immune system less effective, in fighting HPV infections associated with genital area (especially warts) as well as oropharyngeal.

Alcohol

Usage of alcohol is a potent intermediator of immune function which may lead to the breakdown of the immune system, thus leading to increased vulnerability to different chronic and infectious infections. The response to the entry of the pathogen inside the body is divided into two phases: the first phase (an inflammatory reaction) which provides protection against the immediate effects of the infection, and the second phase, where the pathogen develops immunity against the infection. Alcohol consumption can interfere with both phases of the immune response. According to literature; high intake of alcohol is associated with an increased risk for HPV infection with multiple HPV types, which can lead to higher cervical/anal lesion and more common genital warts.

Nutritional factors

For HPV associated disease, an increased risk is reported in patients with deficiencies of vitamin A, β carotene, vitamin C, and folic acid. No data is available on the influence of antioxidants or folic acid, on genital HPV infections. In vitro, bovine papillomavirus (BPV) containing mouse cells, removed the virus with an all-trans retinoic acid treatment, and cell transformation was reversed. However, in rabbits, infected with cottontail rabbit papillomavirus (CRPV) vitamin A treatment leads to regression of papillomas.
2.9. Trends in Human papillomavirus types distribution

Molecular studies have shown that, HPV 16 and 18 are the two most common highly oncogenic types, found in invasive cervical cancer, and among these two; HPV 16 is more frequent. On the other hand, genital warts and benign cervical lesions are associated with 11 low risk HPV types. Among these types, 90% of the genital warts are caused by HPV types 6 and 11. However, HPV is also detected in healthy women, and in women with benign cervical cytology.

Hospital based studies in India, show a prevalence ranging from 9.9-16.6%, among women with benign cervical cytology. The prevalence was higher among high-risk categories, such as, 25% in commercial sex workers, 32.3% at the urban slums in Mumbai, and 41.7-56% in HIV positive women. A meta analysis by Bhatla et al., reported that, there was no significant difference in the prevalence of HPV infection in northern and southern parts of India. However, HPV types 16 and 45 were more prevalent in North India, while, HPV 35 was the prevalent type in South India.

A prospective study from Delhi reported a higher persistence for high-risk HPV type, and the highest rate of persistence was found in HPV types 16, 45, 67, 31, 51, 59. Among the high-risk types, the mean duration of persistence due to HPV 16 was 12.5 months. This was the same as reported by Moscicki, (2005), among adolescents in the US. Prevalence of HPV 18 was greater than that of HPV 16, although, HPV 16 was associated more frequently with high-grade squamous intraepithelial lesion cytology. The incidence rate of HPV infection among healthy women in a slum in Delhi, was found to be 5 per 1,000 women. In young, HPV negative women, the cumulative incidence of a first HPV infection is estimated at 32% after 24 months and 43% after 36 months.

In Maharashtra, the high-risk HPV's were found to be associated with many risk factors, such as, increasing age, low education level, manual work, early sexual debut, etc. However, in Eastern India, the risk of HPV infection was found to be higher in married women, and in women with parity >5.

2.9.1. Global trends in Human papillomavirus infection and cervical cancer
International agency for research in cancer (IARC) - (GLOBOCAN, 2002), have shown that 5.17% of all cervical cancers, were attributed to HPV infection. The incidence rate (global) of cervical cancer, was 16 per 1,00,000 women in 2002, and estimated, 4,93,000 new cases and 2,74,000 deaths with more than 83% cases, occurring in developing countries. However, in 2004, among women, cervical cancer was the 4th most common cause of death, with 4,89,000 new cases reported and 2,68,000 deaths (3.6% out of 7.4 million cancer deaths).

Fig. 2.4 Cervical cancer - estimated incidence, mortality and prevalence worldwide in 2012
(This picture is adopted from: GLOBOCAN 2012, IARC, WHO)

A population based survey in Bangladesh, (2014), has concluded that even in Bangladeshi women, prevalence of HPV infection was found to be similar to other regions of Asia. However, type- specific patterns were different. Whereas, a study on prevalence and type distribution of high-risk HPV infection in genital warts of Korean men found to be significantly associated with the prevalence of high-risk HPV infection. Although, the rate of anal cancer is higher among women than among men. HPV type distribution among women with or without cervical cancer around the world is shown in Table 2.4.
### Table 2.4 Distribution of HPV types in different parts of the world

<table>
<thead>
<tr>
<th>Authors, Year</th>
<th>Place</th>
<th>Methodology</th>
<th>HPV types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klaes et al., (1999)</td>
<td>Germany</td>
<td>RT-PCR Amplification. Hybridization, Cloning, and Sequence analysis.</td>
<td>HR-HPV types 16 and 18</td>
</tr>
<tr>
<td>Schmeink et al., (2011)</td>
<td>The Netherlands</td>
<td>PCR-fragment assay</td>
<td>HPV 16 (30.2%), HPV 51 (19.1%), HPV 31 (16.6%), HPV 52 (14.9%) and HPV 18 (11.1%).</td>
</tr>
<tr>
<td>Jia et al., (2015)</td>
<td>China</td>
<td>Liquid-based Thin Prep Pap test and the HC-II based HR-HPV DNA test, with or without a colposcopic examination.</td>
<td>Mixture of probes used that detected HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.</td>
</tr>
<tr>
<td>Park et al., (2013)</td>
<td>Korea</td>
<td>PCR based DNA microarray system, the HPV DNA chip.</td>
<td>HPV 16 (10%), followed by 18 (6.7%) and 52 (5%).</td>
</tr>
<tr>
<td>Miranda et al., (2013)</td>
<td>Brazil</td>
<td>RT-PCR for isolation RFLP for typing</td>
<td>In low-risk types, HPV 6 infected samples were associated with clearance, while HPV 11, 61, 72, or 81 infected samples were persistent in the follow-up.</td>
</tr>
<tr>
<td>Nahar et al., (2014)</td>
<td>Bangladesh</td>
<td>PCR</td>
<td>HPV16, 66, 18, 45, 31 and 53.</td>
</tr>
<tr>
<td>Park et al., (2014)</td>
<td>Korea</td>
<td>PCR</td>
<td>HPV16 (6.8%), HPV33 (4.5%), HPV18 (2.3%), and HPV68 (2.3%).</td>
</tr>
<tr>
<td>Authors</td>
<td>Country</td>
<td>Methodology</td>
<td>Prevalence</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>Garolla et al., (2014)</td>
<td>Italy</td>
<td>INNO-LiPA HPV Genotyping Extra assay -Pap Smear Cytology -Counseling</td>
<td>HPV 6, HPV 11, and HPV 16</td>
</tr>
<tr>
<td>Cho et al., (2015)</td>
<td>Korea</td>
<td>Liquid-based Papanicolaou test, hybrid capture 2 tests, AnyplexTM II HPV 28 Detection, colposcopic biopsy</td>
<td>HPV 16 is the most persistent HPV genotypes.</td>
</tr>
<tr>
<td>Kim et al., (2012)</td>
<td>Korea</td>
<td>Liquid hybridization and PCR</td>
<td>Prevalence of genital low-risk HPV (6 and 11) was 4.9%.</td>
</tr>
<tr>
<td>Wilson et al., (2013)</td>
<td></td>
<td>GST fusion protein multiplex serology assay</td>
<td>HPV 16, 18, 31, 33, 35, and 45</td>
</tr>
<tr>
<td>Delere et al., (2014)</td>
<td>Germany</td>
<td>PCR</td>
<td>HPV 16 (19.5%) most prevalent type.</td>
</tr>
<tr>
<td>Boers et al., (2014)</td>
<td>The Netherlands</td>
<td>DNA methylation analysis</td>
<td>HR-HPV</td>
</tr>
<tr>
<td>Siddiqa et al., (2014)</td>
<td>Pakistan</td>
<td>PCR</td>
<td>HPV 18 (25.97%), HPV 16 (24.68%) and HPV 16 and 18 both (40.26%).</td>
</tr>
<tr>
<td>Ortiz et al., (2013)</td>
<td>Puerto Rico</td>
<td>PCR</td>
<td>HPV-16, 51, 56, and 90/106</td>
</tr>
</tbody>
</table>
A wide variation has been observed in the reported indices, apart from differences in diagnostic and reporting patterns. The two major factors contributing to the variation in reported incidences are, differences in sexual practices, and differences in access to organized cervical cancer screening programmes. In the etiology of cervical cancer, it is now evident that, the sexual behavior patterns, such as: ages at first intercourse, and the number of lifetime sexual partners of the woman and of her husband, play an important role. These practices determine the woman’s risk for acquiring sexually transmitted disease. Thus, certain sub-groups, such as nuns and groups with strict practices of abstinence and monogamy have long been noted to have very low rates of cervical cancer.214 In contrast, populations with higher rates of sexually transmitted diseases, with extra-marital sexual activities, have higher chance of acquiring cervical cancer.215 This alarming situation in the coming years for Papillomaviruses, has lead molecular virologists worldwide to go deep into pathogenesis of HPV, and bring out solutions to its therapeutic potential.

2.9.2. Trends in India

Cervical cancer constitutes about 15-51% of all female cancers, and its incidence ranges from 17.2 to 55 per 1,00,000 women in different regions of India. Approximately, 80% of invasive cervical cancer, including CIN 3, and 50% of CIN 2 lesions are infected by high risk oncogenic HPV types, i.e., type 16 or 18.216 In the absence of a nationwide screening program, there are disparities in screening, treatment, and also survival. However, individuals who are at the highest risk of developing cervical cancer, such as old age and poor women, are least likely to undergo screening. Opportunistic screening in various regions of India, varied from 6.9%, 9-0.006% and 0.002% in Kerala, Maharashtra and Tamil Nadu, respectively.195

In India, 85-90 % of cervical cancer cases are SCC, and HPV 16 is the most prevalent type among them, compared to other parts of the world, where, the proportion of HPV16 is much lower, when both HPV16 and 18 are considered.85,196,216 HPV type 16 alone in cervical cancer is 70-90%, while, occurrence of HPV type 18, varies from 3-20 %. Other high risk HPV types such as: HPV 45, 33, 35, 52, 58, 59, and 73, have also been reported, but they constitute only a minor group.185,217
The prevalence of HPV 16 in a national mapping study, was found to be 88% (highest) in Chennai, whereas, only 14.2% in Jammu and Kashmir (lowest).\textsuperscript{218} It is found that, the peak of infection was in the age group of 26-35 years, whereas, in western countries infection was common in age group of 18-25 years.\textsuperscript{219, 220}

The role of HPV, as a direct cause of cervical cancer is shown in a study - conducted at Banaras Hindu University, Varanasi, India, - suggesting an urgent need of screening programs, and HPV vaccination in women with low socioeconomic status, and those residing in rural areas.\textsuperscript{221} In a community based study,\textsuperscript{222, 40} rural women from Karnataka, are totally unaware of HPV infection and its health effects. Table 2.5 shows the HPV type distribution among women with or without cervical cancer in India.

\textit{Table 2.5 Distribution of HPV types in various parts of India}

<table>
<thead>
<tr>
<th>Authors, Year</th>
<th>Place</th>
<th>Methodology</th>
<th>HPV types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sowjanya \textit{et al.}, (2005) \textsuperscript{\textsuperscript{85}}</td>
<td>Hyderabad</td>
<td>Primary screening by Digene HC 2 assay and further analyzed using the Roche PCR-based line blot for genotype determination.</td>
<td>HPV 16 (66.7%), HPV 18 (19.4%), HPV 33 (5.6%), HPV 35 (5.6%), HPV 45 (5.6%), HPV 52 (2.8%), HPV 58 (2.8%), HPV 59 (2.8%) and HPV 73 (2.8%).</td>
</tr>
<tr>
<td>Bhatla \textit{et al.}, (2006) \textsuperscript{185}</td>
<td>Delhi</td>
<td>PCR by Reverse line blot hybridization assay.</td>
<td>HPV 16 (73.6%) HPV 18 (14.2%) and HPV 45 (11.3%).</td>
</tr>
<tr>
<td>Franceschi \textit{et al.}, (2003) \textsuperscript{216}</td>
<td>Chennai</td>
<td>Using general primer mediated GP5+/6+ PCR.</td>
<td>23 different HPV types were identified. HPV 16 was the most common type followed by HPV 18 and 33.</td>
</tr>
<tr>
<td>Srivastava \textit{et al.}, (2014) \textsuperscript{221}</td>
<td>Varanasi</td>
<td>PCR</td>
<td>HPV 16 was the most common type followed by HPV 18</td>
</tr>
<tr>
<td>Peedicayil \textit{et al.}, (2006) \textsuperscript{217}</td>
<td>South and East India</td>
<td>Line blot assay</td>
<td>HPV 16 (60%) and HPV 18 (14%) were the most frequent types, but 16 other types (26, 31, 33, 35, 42, 45, 51, 52, 53, 56, 58, 61, 62, 64, 81, and 82) also identified.</td>
</tr>
</tbody>
</table>

\textbf{2.10. Diagnosis of Human papillomavirus infection}
Serological diagnosis is unreliable, as it cannot distinguish between present and past infections, and conventional cell cultures are not useful, because HPV cannot be grown artificially. Therefore, accurate diagnosis of HPV infection can be done by the detection of viral DNA. Additionally, along with detection of HPV DNA, there should be an inclusion of genotyping as well. The important methods to diagnose HPV infection are: colposcopy and acetic acid test, biopsy, DNA test (PCR, Southern blot hybridization, In situ hybridization), and Pap smear.

A. Colposcopy and acetic acid test

Colposcopy is a procedure performed by specially trained clinicians as an outpatient procedure, using a low powered microscope, the colposcope. This procedure is performed after the application of acetic acid, to examine the cervix, vagina and vulva, to collect biopsy material. Greater abnormalities of these parameters are related to severity of the lesions.

Acetic acid test is a method in which 3-5% acetic acid is applied for 5-10 min over the suspected lesions, to enhance the visibility of lesions, by turning them into white. The acetic acid test should not be used for routine screening. It can be used for visualizing subclinical genital HPV associated lesions, identifying lesions for target biopsy, and for demarcating lesions during surgical therapy.

B. Biopsy

Biopsy plays a key role in the colposcopy, as the treatment depends upon on the severity of the biopsy sample, and therefore, the treatment is recommended only when the results show any signs of precancer or cancer. The most characteristic feature in genital warts is the presence of koilocytes, which are, mature squamous cells with clear perinuclear zone. The nuclei of koilocytes may be enlarged, and hyper chromatic, double nuclei are seen often as well.

C. DNA techniques
Initially, HPV detection was done using direct probe hybridization such as dot blot and Southern blot. Besides, being labor intensive and time consuming, they had low sensitivity, and require large amounts of DNA in clinical samples. The established routine methods are as follows:

1) **Polymerase chain reaction**

It is a process of selective target amplification, in which, the HPV sequence is exponentially amplified and produces billion copies from a single HPV DNA molecule, at the end of 30 cycles.\textsuperscript{229} For the detection of HPV, primers used are, those which target the viral capsid L1 gene (consensus primer set include PGMY09/11, GP5+/6+, and SPF10), and those which detect many HPV sub types in one amplification.\textsuperscript{231-233} Type specific PCR, as the name implies, amplifies a single genotype of HPV by targeting a type specific DNA sequence. For type specific PCR, several repeats of PCR may be necessary, in order to determine the specific sequence existing in the sample.\textsuperscript{233} Various applications of target amplification for HPV detection and genotyping are discussed below.

a. **Real time PCR**

For the detection of HPV DNA, real-time PCR is available, which is a highly sensitive target amplification technique. Fluorescent probes are combined with PCR primers, allowing for accurate quantification of virus present in a sample. HPV viral load estimation is a particular advantage of real-time PCR, using the nuclear genome to control the cellular content of the sample.\textsuperscript{231}

b. **Multiplex HPV genotyping kit**

Another novel genotyping test is the Multiplex genotyping kit (Multimetrix, Heidelberg, Germany), which is a PCR based fluorescent bead assay, which can detect 24 low and high risk HPV types. The PCR products are mixed with the multiplex HPV genotyping kit bead mix. This bead mix has 26 beads attached to 24 HPV probes, 1 β-globin probe, and 1 control probe. After hybridization of the PCR products, they are labeled; using R-phycoerythrin marked streptavidin, and read on the luminex analyzer. The individual beads have the ability to differentiate the various types of HPV. Currently, the multiplex HPV genotyping kit, is only available for
research purposes, but it has shown a high sensitivity with applications in large scale epidemiological studies and potential future use in routine diagnostics of HPV.\textsuperscript{234}

2) Hybrid capture HPV DNA test 2 (HC2)

Hybrid capture HPV DNA test 2, in conjunction with the Pap test, is now approved by the Food Drug Adinistration.\textsuperscript{235} The sensitivity and specificity of FDA approved HC2 is almost comparable with PCR based detection methods, as it can detect as little as 1 pg of HPV DNA/mL. The biggest advantage of this test is being very easy to handle and good reproducibility of results. However, the exact HPV type cannot be identified, but low-risk and high-risk HPV genotype groups can be detected.\textsuperscript{229}

3) Reverse line blot and linear assay

The reverse line blot assay from Roche Molecular Systems (Alameda, CA), was one of the first widely used prototype methods. The line blot assay uses L1 consensus primer based PCR, with PGMY 09/11 primers. Probes for multiple HPV types are fixed on a membrane strip, and the PCR product is hybridized to the strip, followed by visual detection. The assay detects 27 different HPV types, and the extended edition adds 11 low-risk types, which include 61, 62, 64, 67, 69 to 72, 81, 82, and 89.\textsuperscript{233} The line blot assay is a research use only test, and the original assay is now commercialized as the linear assay HPV genotyping test.\textsuperscript{234} The results are read with the unaided eye, based on a visible band in specific areas of the hybridization strip.\textsuperscript{236}

4) Amplicor HPV

It is a PCR based kit, that can identify 13 high-risk HPV types, by amplifying target DNA with the help of PCR, followed by nucleic acid hybridization.\textsuperscript{237} This test can detect only presence or absence of HPV, but is unable to identify HPV genotypes. To perform this test, a 96 well microtitre plate and 250 µL of sample is required. However, this method is still not approved by FDA for commercial use.\textsuperscript{237}

5) PapilloCheck

Recently, a commercial DNA based test, called PapilloCheck (Greiner Bio-One, Monroe, NC), is now available for HPV genotyping. This test can identify 24 types of low and high risk HPVs,
from 12 samples at a time, and helps to eliminate false-positive and false-negative results. Genotyping with this method is based on PCR amplification of the E1 gene by a group of new E1-specific primers, followed by hybridization to a DNA chip with immobilized HPV oligoprobes. A laser scanner is used to detect excitation from fluorescent labeled probes, which bind to the HPV primers.\textsuperscript{234}

6) CareHPV

As there is a lack of adequate cervical cancer screening in developing countries, Qiagen has developed a test, called CareHPV, which is a spin-off of Hybrid capture2. CareHPV takes roughly 2.30 hours, and is designed to be used by any minimally trained individual, in less than ideal conditions seen in developing countries. It includes its own water source, allows flexible temperature ranges, and requires no expensive equipment. The short run time allows clinicians to follow-up with patients, within the same visit, thereby, increasing the level of subsequent care. CareHPV has been shown to be 90\% accurate, and has been made much more affordable.\textsuperscript{238}

Currently, there are ongoing pilot studies evaluating CareHPV, in different parts of the world.\textsuperscript{234}

7) PCR and restriction fragment length polymorphism (PCR-RFLP)

After amplification, with the help of restriction enzymes, the sequence composition of a PCR product can be investigated. Digestion of PCR products with restriction endonucleases generates a number of fragments, which can be resolved by gel electrophoresis, yielding a particular banding pattern. This method is straight forward, but labor intensive.\textsuperscript{231}

8) Direct sequence analysis of PCR products

With the help of HPV sequence, the genotype can be extrapolated by two methods. First, the sequence can be used to interrogate a sequence database, using a homology search. Extensive databases are available on the internet and can be freely accessed at http://www.ncbi.nlm.nih.gov. BLAST software \textsuperscript{239} permits fast homology searches of a sequence within a continuously updated sequence database.
Secondly, phylogenetic analyses can be performed. The novel sequence can be used in a multi-sequence alignment with, a known set of HPV sequences, which represent different HPV genotypes. Based on the sequence alignment, a phylogenetic tree can be constructed, providing a graphical representation of the evolutionary relationships between the detected sequence and reference sequences, and a genotype can be deduced. It should be noted that, the formal classification of genotypes is entirely based on sequence analysis of the viral genome, whereas, genotyping of clinical samples is performed by analysis of only a limited, but representative part of the genome.  

D. Pap smear or Pap test

It is the most common screening test used in most of the developing countries, including India. This test was first described by Papanicolaou and Traut, (1928). Not only, pre-malignant and malignant changes can be detected, but also viral infections, like HPV infection and Herpes, can also be detected. If the PAP result comes positive, then further confirmatory tests like, colposcopy, cervical biopsy, and DNA tests like PCR has to be performed.

2.11. Clinical utility of molecular Human papillomavirus diagnosis

Over the past years, there has been a revolutionary change in the diagnosis of HPV infection, following the development of highly sensitive DNA detection assays. However, it requires a careful laboratory validation of the test results, and the interpretation should be done with utmost care. To compare the various diagnostic tools, a well characterized international quality control panels are required. Further, assessment is required for the implications of HPV DNA detection for the patient management. Furthermore, previous evidences suggest that, the presence of multiple HPV genotypes may reflect repeated exposure, and may relate to increased risk for disease progression. Specific molecular tools will be required, because of the extensive genetic heterogeneity of HPVs, and the possible clinical relevance of specific subtypes. Novel low or high density DNA probe tests (DNA chips), may provide a useful technology for such studies.

2.12. Prevention of Human papillomavirus infection

In the prevention of disease there are two approaches, i.e., primary and secondary, which are discussed below.
Primary Prevention

Primarily, the disease can be prevented by eliminating the risk or causative agent before it has been established. In the case of cervical cancer, vaccination to prevent high risk HPV infections or male circumcision, to reduce the likelihood of chronic HPV infection and transmission, are both examples of primary prevention approaches. Unlike, primary prevention, secondary prevention methods, do not seek to entirely prevent disease risk, but to detect early pathological changes through screening, before clinical symptoms have appeared.242

Secondary Prevention

Secondary, by early detection of HPV, through screening and treatment of precancerous lesions, prevention of cancer of cervix uteri can be achieved. Screening is considered to be the most affordable, and sustainable approach, to prevent cancer of cervix. Furthermore, primary approaches to prevent cervical cancer are of no use for women, who are already infected with HPV and/or at risk for developing cervical cancer.242

2.13. Prevention of cervical cancer

Vaccines

Two HPV vaccines are licensed in the US: a quadrivalent vaccine (Gardasil, Merck and Co, Inc.), and a bivalent vaccine (Cervarix, GlaxoSmithKline). Both the vaccines are recombinant Vaccine, as they are composed of virus-like particles (VLPs) prepared from recombinant L1 capsid protein of the targeted HPV types. Cervarix, is directed against two oncogenic types (HPV 16 and 18) whereas, Gardasil, is directed against four HPV types, i.e., two oncogenic types (HPV 16 and 18) and two non-oncogenic types (HPV 6 and 11). Both the vaccines are prophylactic, and not therapeutic, as they are ineffective for HPV related disease progression.134

Gardasil was licensed by the FDA, for use in females (2006) and males (2009) both, aged between 9 - 26 years. Whereas, Cervarix was licensed by the FDA in 2009, for use in females aged between 10-25 years.243, 244

Clinical trials in >18,000 females aged between 15-25 years for Cervarix, and >20,000 females aged between 16–26 years for Gardasil, have demonstrated high levels of efficacy for both vaccines in preventing cervical pre-cancers (CIN 1,2 and 3) caused by the targeted HPV types in females, inexperienced to vaccine type infection at the time of vaccination.245, 246 Gardasil has
also demonstrated high efficacy against HPV 6/11 related genital warts, HPV 16/18 related vaginal and vulvar pre-cancer lesions, and HPV 16/18 related anal precancers (males).\textsuperscript{245}

Immunogenicity and safety studies were conducted in females aged between 9–15 years (quadrivalent vaccine),\textsuperscript{247} and females aged between 10-14 years (bivalent vaccine)\textsuperscript{248} to bridge the antibody titers in females in the efficacy trials. After vaccination for both vaccines, over 99% of the study participants developed antibodies, and it was found that the titers were higher in young girls than in older females, who participated in the efficacy trials.

Each dose (0.5 mL) of Gardasil contains 20 μg HPV 6 L1 protein, 40 μg HPV 11 L1 protein, 40 μg HPV 16 L1 protein, and 20 μg HPV 18 L1 protein. The VLPs are adsorbed on 225 μg amorphous aluminum hydroxyphosphate sulfate adjuvant (alum). Whereas, each 0.5 mL dose of Cervarix contains 20 μg HPV 16 L1 protein and 20 μg HPV 18 L1 protein. The VLPs are adsorbed on 500 μg aluminum hydroxide and 50 μg 3-O-desacyl-4’ monophosphoryl lipid A adjuvant.\textsuperscript{134}

\section*{2.14. Barriers to cervical cancer control programs}

Although, the importance and effectiveness of cervical cancer prevention through screening is demonstrated, and the sociodemographic populations of women least likely to participate in screening have been enumerated, the underlying reasons to explain why majority of women are not utilizing available screening services have not been well described.

One of the major barriers in the detection of HPV mediated diseases and cervical cancer; especially in developing countries, are the cost and technical setting requirements.\textsuperscript{249} Therefore, there is urgent need to develop less expensive and rapid HPV tests which are easy to use in the field settings.\textsuperscript{250}

Second reason could be acceptability of HPV screening as cervical cytology requires insertion of vaginal speculum for examination which is a major barrier in the screening. Therefore, a non cytologic method of screening may resolve the under screening problem in developing countries or a self collected screening method can also increase the screening practice in many resource poor areas where there are limited numbers of clinicians trained in performing speculum examination.\textsuperscript{250}

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Lack of awareness and knowledge regarding HPV infection and cervical cancer among women is the third and major reason in India.\textsuperscript{251-257} However, other factors such as: socioeconomic limitations and lack of nationalized guidelines and policies, also contribute towards the barriers for cancer screening programmes.\textsuperscript{258}

2.15. Limitations of current literature and areas for future study

Piyathilake and colleagues, (2016), only compared HR-HPV positive patients with \textbf{CIN 1} to those with \textbf{CIN 2} and \textbf{CIN 3}, but lacked a healthy control population.\textsuperscript{259} Whereas, Audirac-Chalifour and colleagues, (2016), defined women as normal controls, that had negative cytology and colposcopy, irrespective of HPV status.\textsuperscript{260} Therefore, studies must be appropriately designed to permit accurate interpretation of data, and ensure any observed changes in vaginal microbial communities are directly associated with the pathology.

Based on studies involving very frequent testing over a short space of time, it has now been shown that HPV status can alter quickly over a short period of time. Previously, regarded as a viral infection which simply causes transient infections or persists as a chronic infection.\textsuperscript{261, 262} Whether, this is due to detection and re-detection of low-level persistent infections, due to wavering loss and regained immune tolerance, rather than true reinfection, is currently unclear.

Furthermore, alternative HPV tests for detecting HPV DNA, such as mRNA and E6/E7 levels, in future may help answer these important questions.\textsuperscript{263} Recently it is suggested that, the immune system contributes as little as 20\% towards viral clearance, and that stem cell stochasticity plays the biggest role, based on the integration of epidemiological data with mathematical cellular modeling.\textsuperscript{264} This model may also explain the concept of latency and thus fluctuating HPV status.

2.16. Rationale of exploratory research on Human papillomavirus and cervical cancer in Mangalore

It is estimated that, around 75\% of sexually active women are infected by Human papillomavirus (HPV), as it is one of the most common sexually transmitted pathogens. Cervical cancer remains a leading cause of cancer death among women, living in the developing countries. But, because of the less awareness regarding this infection, and malignancy caused by HPV, and its associated
mortality is increasing. Even though the HPV vaccines are available, their spectrum of prevention is limited, as they can prevent infection by the major types of HPV only. However, cervical cancer may be caused by the other genotypes of HPV as well. Hence, there is a need to rely on early detection of infection by various screening methods and moreover, there is no literature available regarding the circulating genotypes of HPV among women, in and around Mangalore region. Therefore, the present study was conducted to screen the women - attending OBG OPD at Yenepoya Medical College Teaching Hospital, with any gynecological problem - for the presence of HPV DNA, and to genotype the detected DNA, so as to understand the circulating genotypes of HPV among these women patients.